Visualizing and quantifying the suppressive effects of glucocorticoids on the tadpole immune system in vivo

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Schreiber AM. Visualizing and quantifying the suppressive effects of glucocorticoids on the tadpole immune system in vivo. Adv Physiol Educ 35: 445– 453, 2011; doi:10.1152/advan.00001.2011.—A challenging topic in undergraduate physiology courses is the complex interaction between the vertebrate endocrine system and the immune system. There are relatively few established and accessible laboratory exercises available to instructors to help their students gain a working understanding of these interactions. The present laboratory module was developed to show students how glucocorticoid receptor activity can be pharmacologically modulated in *Xenopus laevis* tadpoles and the resulting effects on thymus gland size visualized and quantified in vivo. After treating young tadpoles with a cortisol receptor agonist (dexamethasone) for 1 wk, students can easily visualize the suppressive effects of glucocorticoids on the intact thymus gland, which shrinks dramatically in size in response to this steroid hormone analog. However, the suppressive effect of dexamethasone is nullified in the presence of the glucocorticoid receptor antagonist RU-486, which powerfully illustrates the specific effects of glucocorticoid receptor inhibition on the immune system. Image analysis and statistics software are used to quantify the effects of glucocorticoid modulation on thymus size.

*Xenopus;* thymus; endocrinology

THE INTERACTION between the vertebrate endocrine system and the immune system is a fascinating and complex topic for undergraduate physiology students. Of particular importance is the notion that the neuroendocrine stress response modulates immune activity (Fig. 1) (for reviews, see Refs. 2, 15, and 20). One of the functions of glucocorticoids, such as cortisol, is to minimize the inflammatory response to injury. Cortisol has diverse medical applications: it is used to reduce inflammation caused by allergic reactions, suppress autoimmune disorders, and reduce white blood cell counts in patients with leukemia. However, the immunosuppressive effects of elevated cortisol levels, whether administered by a physician or induced by chronic stress, can also be detrimental to patients already suffering from illness. As students are sometimes painfully aware, prolonged levels of stress (such as studying for final exams) can even increase the susceptibility of otherwise healthy people to illness. Despite the fact that most undergraduate physiology textbooks cover endocrine-immune interactions at some level, very few published laboratory exercises are available that show students how to directly and specifically modulate the glucocorticoid stress pathway and visualize the subsequent effects on the immune system. Several excellent undergraduate-level laboratory exercises in stress endocrinology have been developed, including the quantification of the glucocorticoid response to stress in human saliva (9), the use of live frogs to visualize the effects of hormones (including stress hormones) on melanocyte development (11), and hematological approaches to addressing the effects of environmentally or socially induced stress on blood chemistry and immune cell profiles in fish (7) and mice (3). The present laboratory exercise was designed to show students how glucocorticoid receptor activity can be pharmacologically modulated in tadpoles and the resulting effects on thymus gland size visualized and quantified in vivo.

The vertebrate immune system consists of diverse cell types that are broadly dispersed among many different organs (e.g., the spleen, bone marrow, blood, thymus, and lymph nodes). Although these organs are difficult to view in most living organisms, young *Xenopus laevis* tadpoles are virtually transparent, with the pair of thymus glands easily visible using a stereomicroscope (Fig. 2). The thymus gland is critically important for the maturation of T lymphocytes (T cells) and the production of thymosins, hormones that control the activity of T cells and other immune components. In humans, the thymus is largest and most active in infants and children. Although the thymus begins to atrophy in the early teens and regresses significantly by middle age, residual thymus tissue still facilitates T cell maturation throughout adulthood (for a review, see Ref. 2). Importantly, in mammals, the immunosuppressive effects of elevated cortisol levels are known to cause the thymus to regress in size by promoting thymocyte apoptosis (2, 6, 13). The amphibian thymus is also highly responsive to cortisol, and, during tadpole metamorphosis, elevated glucocorticoid levels cause a significant regression in thymus size as the larval immune system is destroyed and replaced by a new adult immune system (1, 10, 16–18).

Glucocorticoids are a class of steroid hormones produced by the vertebrate adrenal cortex. Diverse factors mediate the production of the glucocorticoid cortisol, such as the circadian clock, dietary status, and (as shown in Fig. 1) stress (8). Like all steroid hormones, the classical actions of glucocorticoids are mediated by binding to cytosolic or nuclear-localized receptors that ultimately function as gene transcription factors (for reviews, see Refs. 14 and 15). Nongenomic, rapid-response pathways that function via cell membrane-localized steroid hormone receptors have also been described (12). The glucocorticoid receptor agonist dexamethasone (Dex) is a potent synthetic member of the glucocorticoid class of steroid drugs. It is 20–30 times more potent than the naturally occurring hormone hydrocortisone and acts as an anti-inflammatory and immunosuppressant agent. The glucocorticoid and progesterone receptor antagonist RU-486 (mifepristone) is a synthetic modified steroid that competitively inhibits the action of receptor ligands (5). The antiguclocorticoid effects of RU-486...
are under investigation as treatments for hypercortisolism (Cushing’s syndrome) (5). The chemical structures of both Dex and RU-486 are remarkably similar to that of endogenously produced cortisol (Fig. 3). Both Dex and RU-486 have been effectively used to either induce (Dex) or block (RU-486) the glucocorticoid immune response in tadpoles (1, 16, 17). The ease of treating tadpoles by merely adding these pharmacological analogs to their water, in combination with the clear visibility of the thymus in vivo, makes the tadpole thymus an ideal model to observe endocrine-immune interactions, particularly in an undergraduate laboratory setting.

In addition to its antiglucocorticoid effects, RU-486 is also a potent antiprogesterone used clinically for the induction of abortions (5). Progesterone is a steroid hormone that has two functions: 1) it can serve as a substrate for the synthesis of other steroid hormones and 2) it can bind to its own receptors, which are known to be located, in part, within the reproductive organs of adults. Although progesterone receptor distribution and function have not been widely studied in tadpoles, emerging evidence from adult mammals suggests that progesterone receptors may also be located in nonreproductive regions of the brain, urinary tract, skin, and gastrointestinal organs (4, 19).

The objectives for this laboratory module are as follows:
- Foster an understanding of pharmacological agonism and antagonism of steroid hormone receptors.
- Visualize the suppressive effects of cortisol on thymus gland size in living tadpoles.
- Capture and quantify visual data from live animals using microscopy and image-analysis software.
- Analyze and assimilate data into tabular, graphic, and image formats.

MATERIALS AND METHODS

Microscopy and Image-Analysis Equipment Needed

The following equipment is necessary for this laboratory module:
- A stereomicroscope with a range of at least ×1–10 magnification (we use a Nikon SMZ 1500). A stereomicroscope equipped with a trinocular C-mount for a camera is desirable, but not necessary.
A digital camera. This can be any camera capable of adapting to a methasone (Dex; a synthetic cortisol receptor agonist), and RU-486 (a synthetic cortisol receptor antagonist) are similar.

- A stage micrometer or ruler. This will be used to calibrate distance with "eyepiece microscope camera."
- ImageJ software. This image-analysis software and the instruction manual, developed by the National Institutes of Health, can be freely downloaded onto any computer platform (http://rsbweb.nih.gov/ij/).
- A thermometer for checking the water temperature.
- A stage micrometer or ruler. This will be used to calibrate distance with ImageJ.
- Statistical analysis and graphing software. This can be any software capable of running one-factor ANOVA and pairwise multiple-comparison tests (e.g., Tukey’s test).

Tadpoles and Materials

Numbers of tadpoles and materials needed for the exercise are as follows:

- One hundred X. laevis tadpoles (6 days postfertilization; Xenopus Express no. TAD, $40). Additional sources of X. laevis tadpoles include "Xenopus 1" and "NASCO."
- Dex (D-4902-100MG, Sigma, $57).
- RU-486 (M-8046-100MG, Sigma, $66).
- DMSO (10 ml, no. 89139-666, VWR, $16).
- Ethyl-3-aminobenzoate (anesthetic, TCA1460-005G, VWR, $18).
- One-liter plastic containers (4). This can be any round container with a diameter that is longer than its depth (this ensures a high enough surface area-to-volume ratio such that aeration is not required),
- "Turkey baster" bulb pipettes (4) for transferring tadpoles. These can be purchased anywhere that basic kitchen supplies are sold.
- Two 5-gallon buckets with a lid for storing the newly arrived tadpoles and the 0.1× MMR working buffer (see below).
- A thermometer for checking the water temperature.
- Several disposable plastic 3-ml transfer bulb pipettes.
- Several small plastic petri dishes for microscopy. A convenient size is one made by Fisher Scientific (size: 60×15 mm, no. 08–757-13A).

Reagents to Make and Store in Advance

The following reagents should be made and stored in advance:

- 10× MMR stock buffer [1 liter, composed of 1 M NaCl (58.44 g), 20 mM KCl (1.49 g), 10 mM MgCl2·6H2O (2.03 g), and 50 mM HEPES (11.92 g)]. All ingredients should be dissolved in deionized water and brought to a pH of 7.5 using 10 N NaOH. Store at room temperature.
- 0.1× MMR working buffer (13 liters). This buffer is composed of 130 ml of 10× MMR stock buffer added to 13 liters of deionized water and stored at room temperature in a 16-liter (5 gallon) bucket. This is the water that will be used to treat the tadpoles.
- 0.01 M Dex (dissolved in DMSO) stock solution. Store frozen (−20°C) in aliquots of 225 μl/tube.
- 0.001 M RU-486 (dissolved in DMSO) stock solution. Store frozen (−20°C) in aliquots of 175 μl/tube.
- 1% Ethyl-3-aminobenzoate (dissolved in 0.1× MMR buffer) working anaesthetic. Make at 25 ml and refrigerate at 4°C. This can be stored in solution for 1 mo.

Important Safety Precautions

Pregnant women should NOT handle or be exposed to RU-486 in this laboratory, as it is a potent antiprogesterone that is used clinically to induce abortions (5). Dex, RU-486, and DMSO are toxic compounds that are highly permeable across human skin and can diffuse into the bloodstream readily. These chemicals must be treated with caution: gloves and laboratory coats must always be worn when handling tadpoles immersed in these solutions, and particular care must be exerted during water changes. Solutions containing these chemicals should not be disposed of in a sink; they should be transferred to glass hazardous waste disposal containers according to the safety guidelines of your institution.

Time Frame

This laboratory is designed for students taking an upper-division undergraduate endocrinology or physiology course and can be implemented in two consecutive laboratory periods (2–3 h each) that meet once per week. The procedures accommodate a class size of up to 16 students (four teams of four students). The laboratory time frame assumes that students are already familiar with statistical analysis (one-factor ANOVA), can analyze and interpret data independently outside of class, and are familiar with basic table- and figure-making techniques.

Instructor preparations the day before the laboratory (estimated time: 15 min). The instructor should schedule the tadpoles to arrive the day before the first laboratory session. Upon arrival, the tadpoles and all of their shipping water should be gently transferred to one 5-gallon bucket. Increase the volume of water in the bucket by ~25% by adding 0.1× MMR buffer (stored at room temperature) to it. The tadpole water will likely be much colder than room temperature (they are purposely cooled when shipped), and the animals will require an overnight acclimation period to slowly bring the water to room temperature.

Week 1: introduction, setup, treatment, and ImageJ tutorial (total time: 2.5–3 h). LABORATORY DAY PART 1: DISCUSSION OF THE LABORATORY’S RATIONALE (ESTIMATED TIME: 45–60 MIN). Several problem-solving exercises (see PRELABORATORY PROBLEM-SOLVING EXERCISES) have been designed to foster thought and discussion about the laboratory’s experimental design rationale. Students can work on these before the laboratory or, alternatively, at the start of the laboratory. The answers can be discussed with the instructor as a class before the experiment is begun.

LABORATORY DAY PART 2: STARTING THE EXPERIMENT (ESTIMATED TIME: 30–45 MIN). First, students should sort and transfer the tadpoles into four 1-liter aquaria, each containing exactly 500 ml of 0.1× MMR buffer. Aquarium should be labeled as “control,” “RU-486 (150 nM),” “Dex (2 μM),” and “RU-486 + Dex.” Each student group can be responsible for maintaining one aquarium over the next week.

Second, students can thaw out RU-486 aliquots and add 75 μl RU-486 stock solution to 500 ml of 0.1× MMR buffer in the respective aquaria. Students should add 75 μl DMSO to the control and Dex (2 μM) aquaria.
Important note: Dex is not added to the aquaria on the first day but will be added on subsequent days. The reason for starting RU-486 treatment 24 h before the addition of Dex is to give RU-486 time to bind to the cortisol receptors in the absence of competition from Dex.

Note on daily maintenance: Dex and RU-486 are relatively unstable molecules that degrade at room temperature, and after treatments have been administered, water changes must be conducted every 24 h (see How to perform effective water changes) for 1 wk until the start of the next laboratory session. Treatment with Dex begins the day of the first water change. Students should thaw out Dex and RU-486 aliquots and add 100 µl stock Dex solution and add 75 µl stock RU-486 solution to 500 ml of 0.1× MMR buffer in the respective aquaria. DMSO (75 µl) is added to the control aquarium.

LABORATORY DAY PART 3: TUTORIAL ON MICROSCOPY AND IMAGE ANALYSIS (ESTIMATED TIME: 60 MIN). The students will not begin to collect their data until the next week. However, the instructor can use this time during the first laboratory period to teach the students how to properly use the stereomicroscope, photograph the tadpoles, and use ImageJ to measure thymus size.

For microscopy and photography, the instructor can use 7-day-old tadpoles to demonstrate the methodology for euthanization and image capture (see Tadpole Handling Tips). Three photographs for each tadpole are ultimately needed: 1) one at low magnification (e.g., ×2), where the entire head width is visible so that interocular distance can be determined; 2) one at higher magnification (e.g., ×7) of the left thymus; and 3) one at higher magnification of the right thymus.

For image analysis with ImageJ, ideally, the instructor will demonstrate the use of the ImageJ software to the class using a computer projector. Interocular distance (a measure of “head size”) is obtained from dorsal side tadpole images using the “line tool” (see Fig. 4B). The purpose of recording a measurement of head size is to show that any change in thymus size with treatment is not a result of differential somatic growth rates, in general. The surface area for each thymus is measured using the “polygon tool” to circumscribe the thymus (see Fig. 4C). Since the left and right thymus often vary in size in the same animal, students should quantify the “total” thymus two-dimensional surface area (i.e., collect the sum of the left thymus + right thymus to obtain one value per tadpole). Three measurements for each tadpole will be taken: 1) total thymus surface area (in µm²); 2) interocular distance (in µm); and 3) the thymus size index, a dimensionless value derived from the ratio of the thymus size to the measurement of head size (interocular distance) using the following formula: thymus size index = total thymus surface area/interocular distance² × 1,000.

After the demonstration of data-gathering methods with ImageJ, students can practice taking these measurements using sample images that the instructor has prepared in advance. Alternatively (and time permitting), students can euthanize and measure 7-day-old tadpoles and use these data as baseline, “day 0” measurements.

Note regarding the tadpole size index: although the tadpoles are all the same age, there is still a large degree of variability in both thymus and body size among the animals. Differences in thymus size must be normalized to variability in body size. There are several different ways to measure body size, including total tadpole length (snout to tail tip length), total tadpole surface area, and total tadpole weights (wet or dry). To meaningfully compare the thymus size-to-body size ratio, the units of measure for the thymus and body must be identical (i.e., if thymus size is measured as µm², then body must also be measured in µm² so that the units cancel out). Therefore, if thymus size is measured as the two-dimensional surface area, then it cannot be normalized to a body size measured by weight. Intercocular distance is an excellent indicator of relative head size, and this can be measured accurately and consistently, even when the tadpole is slightly tilted. Although interocular distance is a linear measurement (i.e., units of µm), it can be transformed into units compatible with surface area (units of µm²) by squaring the interocular distance. Hence, interocular distance can be transformed into a useful body size index suitable for determining the thymus size-to-body size ratio. By multiplying this ratio by a constant, 1,000 (see the above formula), the resulting numbers typically fall within a range of 1–4 (see Fig. 7).

Week 2: microscopy and image analysis (total time: 2–3 h). PART 1: MICROSCOPY (ESTIMATED TIME: 30–45 MIN). Students can work in groups to anesthetize and photograph at least 10 tadpoles/treatment group (i.e., group 1 photographs the controls, group
2 photographs the Dex only-treated tadpoles, group 3 photographs the RU-486 only-treated tadpoles, and group 4 photographs the Dex + RU-486-treated animals; see How to anesthetize and euthanize tadpoles for photography). The image data can be compiled into one computer folder that is made accessible to all students for analysis.

Note regarding the total time estimate: it takes ~30–45 min to photograph a group of 10 tadpoles. Therefore, if only one stereomicroscope/camera setup is available to the laboratory, a total of 2–3 h of microscope time will be needed to collect all of the images.

PART 2: IMAGE ANALYSIS WITH IMAGEJ SOFTWARE (ESTIMATED TIME: 45–60 MIN). After the tadpole images have been collected for one group, those students can begin to use ImageJ to derive length and surface area measurement data for each tadpole. Each student group can measure interocular distance and thymus surface area (Fig. 4) for one treatment group. The three data measurements for each tadpole (total thymus surface area, interocular distance, and thymus size index) can then be compiled onto a common class Excel spreadsheet for statistical analysis outside of class.

Since this image-analysis step cannot be initiated until the data is collected, the number of stereomicroscope/camera setups (above) available may limit the ability of all four groups to complete image analysis by the end of the 3-h laboratory period. However, this analysis can be completed outside of class.

The visual and numerical data obtained in this laboratory provide students with opportunities to assemble and present their data in three different ways: tabular, graphic, and photograph-derived forms. Below (see Postlaboratory Data Analysis and Presentation) are sample questions asking students to present their data in these forms. The resulting tables and figures can be incorporated into a traditional laboratory report or evaluated as a stand-alone assignment. Tables, graphs, and image data can be created using Microsoft Word, Excel, and Powerpoint (respectively) or similar software.

Tadpole Handling Tips

Duration of treatment. The optimal tadpole age to order is 6 days postfertilization because the animals have a residual yolk sac and have not yet started to feed. Treatment of tadpoles should begin no later than 7 days postfertilization. The tadpoles should not be fed before or during the experiment (7–14 days postfertilization), as feeding will produce high variability in tadpole growth rates that can confound statistical analysis due to the relatively small sample size being measured. Since the tadpoles are not being fed, the duration of the experiment should not exceed 7 days, as they will begin to die of starvation after 1 wk.

Minimize temperature stress. During the experiment, water temperatures must fall between 20 and 24°C. Never transfer tadpoles to new water that differs by >1.5°C, as the shock will result in their death after several days. Be sure the 0.1× MMR buffer that the tadpoles are being transferred to is already at the same temperature as the water they are in.

Minimize transfer stress. Never use nets to transfer young tadpoles from one container to another; the stress produced will result in their death after several days. Tadpoles should first be transferred to a beaker (e.g., 100-ml size) using a wide-mouth bulb pipette, such as a turkey baster. Before tadpoles are transferred into their treatment containers, pour out all excess water from the transfer beaker and let the tadpoles slide out into the new water.

How to perform effective water changes. Dex is unstable, and water changes must be performed daily over the course of treatment to ensure a constant exposure (e.g., six water changes over a 7-day treatment period). The following are instructions on how to perform effective water changes:

1. Gently and slowly pour ~85–90% of the water from the tadpole container into another empty container. If tadpoles are accidentally poured out, they can be recovered from the second container using a turkey baster pipette (important note: avoid cross-contamination by using one labeled pipette for each treatment group).
2. Gently and slowly pour exactly 500 ml of fresh 0.1× MMR buffer into the tadpole container.
3. Thaw out aliquots of Dex and RU-486 by placing the tubes in hot water from the faucet for several minutes and then vortex each tube.
4. Using a pipette, add 100 µl of stock Dex solution to the appropriate tadpole container (100 µl of stock Dex solution in 500 ml of tadpole water will yield the final desired 2 µM working solution) and add 75 µl of stock RU-486 solution to the appropriate containers (75 µl of stock RU-486 solution in 500 ml of tadpole water will yield the final desired 150 nM working solution).

How to anesthetize and euthanize tadpoles for photography. The following are instructions on how to anesthetize and euthanize tadpoles for photography:

1. Use a clipped disposable plastic transfer pipette to capture a single tadpole from its treatment container.
2. Transfer the tadpole to a small (e.g., 30 ml) petri dish filled approximately half full with 1% ethyl-3-aminobenzoate anesthetic solution. The tadpole will become immobile after a few seconds.
3. Use small pipette tips or pins to move the tadpole to the center of the dish. Ensure that the dorsal side of the tadpole is facing up. Ensure that the animal is completely covered with solution, as any exposed skin will reflect light and overexpose the image.
4. At least three photographs should be taken per tadpole: one image at low magnification (e.g., ×2) of the head (this will be used to derive anterior head surface area) and two images at higher magnification (e.g., ×7) of each thymus gland (these will be used for quantifying thymus gland size; see Figs. 2 and 4). It is important that the magnifications for each photograph are recorded.
5. After photography, the tadpole should be transferred to a small beaker containing 1% ethyl-3-aminobenzoate anesthetic solution. After tadpoles have been euthanized due to anesthetic overdose (this usually takes ~5–10 min; death can be confirmed by absence of a heart beat), the carcasses can be disposed of according to institutional protocols.
6. Photographs of either a stage micrometer or a ruler should be taken for each magnification of the tadpole images. These images of known lengths will be used later to calibrate the ImageJ software to accurately determine thymus surface area measurements.

PRELABORATORY PROBLEM-SOLVING EXERCISES

Problem-Solving Exercise 1

The notion that varying concentrations of the agonist and antagonist will yield different results can be explored by having the students complete the following exercise. This hypothetical dose-response relationship can be explored in actual experiments as an extension to the laboratory or as an independent project.

Determination of optimal concentrations. Suppose you want to determine the effect of glucocorticoid treatment on tadpole thymus size by administering two pharmacological agents: Dex and RU-486. However, you have no idea of the optimal concentrations at which to administer each chemical. What factors must you consider when determining the optimal concentration? Design a pilot experiment that identifies the optimal concentration for each compound and explain your reasoning.

Effect of increasing Dex concentrations. You perform an experiment in which you raise different groups of tadpoles in water containing different Dex concentrations for 1 wk. Draw
glucocorticoid receptors will be saturated with the ligand and theoretically, a concentration of Dex will be reached in which all decreases with increasing Dex concentrations. However, theoretically, a concentration of RU-486 will be reached in which all glucocorticoid receptors will be saturated with the antagonist, resulting in no further effect on thymus size beyond that concentration (see Fig. 5B).

Effect of increasing RU-486 concentrations. You have identified the optimal concentration of Dex that provides the maximum effect on thymus size with minimal mortality using the strategy above. You now perform an experiment in which you raise different groups of tadpoles, all in the same optimal concentration of Dex for 1 wk. However, in addition, you add to each tadpole group a different concentration of RU-486. Draw a graph and label the y-axis as “thymus size” (use relative units of 0 as the lowest size and 100 as the highest size) and the x-axis as “Dex concentration” (use relative units of 0 as the lowest size and 100 as the highest size). Predict how average thymus size will change after a 1-wk treatment with increasing Dex concentrations. Assume, for the purposes of this exercise, that Dex is not lethal and does not affect tadpole growth at any concentration, even the highest concentrations.

Effect of increasing Dex concentrations. Thymus size should decrease with increasing Dex concentrations. However, theoretically, a concentration of Dex will be reached in which all glucocorticoid receptors will be saturated with the ligand and possibly even downregulated, resulting in no further decrease in thymus size beyond that concentration (see Fig. 5A).

Problem-Solving Exercise 1

Determination of optimal concentrations. The optimal concentration of any individual treatment must consider at least three key parameters: 1) the lowest dose that will yield the largest effect on the parameter measured (thymus size), 2) the dose must be sublethal, and 3) the dose should ideally not affect the general size (i.e., growth rate) of the animal. The optimal doses for Dex should first be determined by a series of dose-response experiments. After the optimal dose of Dex has been determined, the optimal dose of RU-486 can then be determined by a series of dose-response experiments in which all groups are also treated with the optimal concentration of Dex.

Problem-Solving Exercise 2

Answers to Problem-Solving Exercise 1

Determination of optimal concentrations. The optimal concentration of any individual treatment must consider at least three key parameters: 1) the lowest dose that will yield the largest effect on the parameter measured (thymus size), 2) the dose must be sublethal, and 3) the dose should ideally not affect the general size (i.e., growth rate) of the animal. The optimal doses for Dex should first be determined by a series of dose-response experiments. After the optimal dose of Dex has been determined, the optimal dose of RU-486 can then be determined by a series of dose-response experiments in which all groups are also treated with the optimal concentration of Dex.

Effect of increasing Dex concentrations. Thymus size should decrease with increasing Dex concentrations. However, theoretically, a concentration of Dex will be reached in which all glucocorticoid receptors will be saturated with the ligand and possibly even downregulated, resulting in no further decrease in thymus size beyond that concentration (see Fig. 5A).

Effect of increasing RU-486 concentrations. Thymus size should increase with increasing RU-486 concentrations. However, theoretically, a concentration of RU-486 will be reached in which all glucocorticoid receptors will be saturated with the antagonist, resulting in no further effect on thymus size beyond that concentration (see Fig. 5B).

The working concentrations for Dex and RU-486 were previously optimized and derived from studies by Rollins-Smith et al. (17, 18), and the frozen stock solutions for treating the tadpoles will have been prepared in advance by the instructor. However, before administering the treatments, students can gain an understanding of how to prepare working solutions from stock solutions of known molarity by completing Problem-Solving Exercise 2.

Problem-Solving Exercise 2

You have purchased 1 mg of RU-486 and 10 mg of Dex from a chemical supplier. You want to make frozen stock solutions for each (RU-486: 0.001 M and Dex: 0.01 M) dissolved in DMSO (a supersolvent). The formula weights for RU-486 and Dex, respectively, are 429.6 and 392.46 g/m.

Creation of the appropriate stock concentrations. What volumes of DMSO need to be added to each chemical vial to create the appropriate stock concentrations?

Determination of the final working concentrations of stock solutions. What volume of each stock solution needs to be added to each appropriate aquarium (assume that the aquarium contains 500 ml of water) to make final working concentrations of 150 nM RU-486 and 2 μM Dex, respectively?

Answers to Problem-Solving Exercise 2

Creation of the appropriate stock concentrations. DMSO at 2.33 ml is added to 1 mg of RU-486 to make a 1 mM (0.001 M) stock solution. DMSO at 2.55 ml is added to 10 mg of Dex to make a 0.01 M stock solution.

Determination of the final working concentrations of stock solutions. Stock RU-486 (0.001 M) solution at 75 μl is added to 500 ml of tadpole water to make a 150 nM working solution. Stock Dex (0.01 M) solution at 100 μl is added to 500 ml of tadpole to make a 2 μM working solution.

Problem-Solving Exercise 3

Answer the following questions regarding this laboratory’s experimental design and analysis:

A. What experimental purpose does each treatment group (untreated control, RU-486 only, DEX only, and RU-486 + Dex) serve? What is the purpose of adding DMSO to the untreated control group?

B. To obtain the best results, it is important that we begin RU-486 treatment 24 h before we initiate Dex treatment. Why is it important that we start RU-486 treatment before Dex? What could happen if we started treating the tadpoles with both RU-486 and Dex at the same time?

C. What is the purpose of measuring thymus size?

D. What is the purpose of measuring interocular distance?

E. Your data will ultimately be transformed into a thymus size index in which thymus surface area is divided by interocular distance. Why does this index provide a more accurate
Table 1. Typical student-derived tabular data

<table>
<thead>
<tr>
<th></th>
<th>Untreated Control</th>
<th>Dex</th>
<th>RU-486</th>
<th>Dex + RU-486</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interocular distance, mm</td>
<td>1.87 ± 0.03*</td>
<td>1.88 ± 0.07*</td>
<td>1.95 ± 0.05*</td>
<td>1.80 ± 0.04*</td>
</tr>
<tr>
<td>Total thymus surface area, μm²</td>
<td>7,455 ± 380*</td>
<td>4,123 ± 199†</td>
<td>7,473 ± 531*</td>
<td>7,560 ± 400*</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 10 tadpoles. Dexamethasone (Dex) treatment significantly reduced tadpole thymus size compared with controls, whereas RU-486 inhibited this suppression. Interocular distance (a measure of head size) was not affected by this treatment. Total thymus surface areas for each tadpole were the sum of the left and right thymus glands. Measurements were derived from tadpoles (14 days postfertilization) after 7 days of treatment with Dex (2 μM) and/or RU-486 (150 nM). Means in the same row with the same superscript (*, †) were not significantly different by one-factor ANOVA and Tukey’s pairwise post hoc tests (P < 0.05).

Fig. 6. Typical student-derived image data of representative thymus glands from tadpoles (14 days postfertilization) after treatment (7 days) with Dex and/or RU-486. Control tadpoles were treated with DMSO solvent only. Working concentrations for Dex and RU-486 were 2 μM and 150 nM, respectively.

Fig. 7. Typical student-derived graph data of thymus size indexes from tadpoles (14 days postfertilization) after treatment (7 days) with Dex and/or RU-486. Dex (2 μM) significantly reduced the tadpole thymus size index compared with controls, whereas RU-486 (150 nM) inhibited this suppression. Thymus size indexes from groups with different letters were significantly different from each other by one-factor ANOVA and Tukey’s pairwise post hoc tests (P < 0.05), n = 10 tadpoles.

portrayal of the effects of treatments on thymus size compared with just evaluating thymus size data alone?

F. After treating the tadpoles under the four conditions for 1 wk, what results do you predict you will see, and why?

G. Suppose you find that treatment with Dex results in a smaller thymus size. Propose at least three different explanations for how the thymus could become smaller.

H. Suppose you find that treatment with Dex results in a smaller thymus size. Does this mean that the tadpole’s immune system function has definitely been inhibited? Explain.

Answers to Problem-Solving Exercise 3

Answers to the above questions are as follows:

A. The untreated group is the baseline comparison that also controls for any DMSO solvent-specific effects on thymus size, mortality, and growth. Treatment with RU-486 alone identifies RU-486-specific effects on thymus size, mortality, and growth. Treatment with Dex alone identifies Dex-specific effects. The RU-468 + Dex combined treatment determines if the effects of Dex on thymus size are in fact mediated at the nuclear transcription level via the Dex receptor and not by some other pathway.

B. By adding RU-486 before Dex, we increase the probability that RU-486 will bind to and “saturate” available cortisol receptors and reduce the ability of Dex to compete for binding sites. If we started both RU-486 and Dex treatments at the same time, some Dex would outcompete with RU-486 for receptor-binding sites, resulting in a weaker antagonistic effect by RU-486.

C. Thymus size will serve as a measurable index of immune status.

D. Interocular distance is a measurement of head size used to determine if treatments affect the general growth of the tadpole.

E. The thymus size index normalizes any differences in thymus size that result from differences in tadpole head size (i.e., larger tadpoles may have larger thymus glands compared with smaller tadpoles).

F. Student responses to this question will, of course, depend on the extent that they have been introduced to the topics of stress-immune system interactions and the mechanisms of hormone receptor agonist/antagonist functions. The two key points the students should grasp are 1) if glucocorticoids
suppress the immune system, it is reasonable to hypothesize that Dex treatment would result in a smaller thymus, and 2) if RU-486 functions as a cortisol receptor antagonist, it should inhibit the suppressive effects of Dex when treated in combination.

G. When considering the thymus gland, it is important to note that the gland broadly consists of two cell categories: 1) a population of thymic stromal cells that forms the main structure of the gland and 2) populations of externally derived lymphocytes of hematopoietic origin that travel to the thymus where they mature into T (thymus-derived) lymphocytes that have multiple roles in the immune response (e.g., function as “cytotoxic T cells” that destroy virally infected cells and tumor cells, “T helper cells” that assist the maturation of B cells into antibody-releasing plasma cells, and “memory T cells” that persist after an infection has been destroyed) (8). Therefore, smaller thymus size could theoretically be attributed to at least several nonmutually exclusive possibilities: 1) reduced cell proliferation, 2) increased cell death, 3) reduced cell size, and 4) reduced migration of externally derived lymphocytes into the thymus gland.

H. Although it is tempting to assume that a smaller thymus would be unable to produce as many mature T cells as a larger thymus, and hence be indicative of reduced immune system function, this cannot be concluded on the basis of thymus size alone. The most convincing way to determine if reduced thymus size corresponds with reduced T cell production is to actually count the number of T cells in the tadpoles, a process that requires the use of cell and molecular biology methodologies.

Postlaboratory Data Analysis and Presentation

The visual and numerical data obtained in this laboratory provide students with opportunities to gain experience in assembling and presenting their data in three different ways: tabular, graphic, and photograph-derived forms. Below are sample exercises asking students to present their data in these forms. The resulting tables and figures can be incorporated into a traditional laboratory report or evaluated as a stand-alone assignment. Tables, graphs, and image data can be created using Microsoft Word, Excel, and Powerpoint (respectively) or similar software.

1. Create a figure that shows a typical image of an untreated X. laevis tadpole head. Denote the location of each thymus gland using arrows or boxes. Be sure to include scale bars and a figure legend. (An example of a typical student-derived figure is shown in Fig. 2.)

2. Create a table showing the following:
A. Mean total thymus (left + right) surface areas (±SE) for each treatment.
B. Mean interocular distance (±SE) for each treatment.
C. Statistically significant differences in size among the different treatments by one-way ANOVA followed by Tukey’s post hoc tests. (An example of a typical student-derived table is shown in Table 1.)

3. Make a four-panel figure that shows one representative high-magnification thymus photograph from each of the different treatment groups. Be sure to include scale bars and a figure legend. (An example of a typical student-derived figure is shown in Fig. 6.)

4. Create a scatterplot or histogram showing the thymus size index for all tadpoles in each of the four treatment groups. An advantage of a scatterplot over a histogram is that within-treatment variability is easier to visualize. Show statistically significant differences in size among the different treatments by one-way ANOVA followed by Tukey’s post hoc tests. Include a figure legend. (An example of a typical student-derived figure is shown in Fig. 7.)

SUMMARY AND EXTENSIONS

This laboratory module provides an opportunity for students to pharmacologically manipulate the glucocorticoid pathway at the whole organism level, deriving data from intact, living vertebrates, and shows students how qualitative observations from captured images can be quantified and measured accurately. The module fosters analytic thinking with statistical analysis and problem sets designed to give students practical experience making stock solutions and diluting them into working concentrations. The exercise promotes various forms of data collection, such as capturing microscope-derived images and basic spreadsheet data entry. The data lend themselves to diverse modes of presentation, including image-derived figures, graphs, and tables.

This laboratory module has been implemented for 4 yr as part of St. Lawrence University’s General and Comparative Endocrinology course. The module provides many opportunities for collaborative approaches to learning and problem solving. For example, I require that my students work in small teams to address the three problem-solving exercises outside of class as well as the statistical analysis, graph, and table synthesis components. Finally, exposure to the elegant tadpole model invariably fosters new student-generated ideas for conducting extensions of this exercise and further independent experiments, such as measuring the effects of environmental endocrine disruptors (such as bisphenol A as well as various pesticides and herbicides) or other hormones (e.g., thyroid hormone or estradiol) on thymus gland development. An advantage of young X. laevis tadpoles is that virtually any water-soluble compound can be effectively administered merely by adding it to the water; the transparent nature of the young tadpole skin allows many different organs to be visualized in vivo. Students can also go beyond live tadpole microscopy and study the cellular morphology of the thymus using histological techniques (e.g., sectioning the thymus onto slides and staining with hematoxylin and eosin).

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

REFERENCES


